

Relationships between DNA Adduct Formation and Carcinogenesis

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An impressive array of evidence has been obtained during the past decade establishing correlations between specific DNA adducts and carcinogenesis. Many of the studies utilized organ specific differences in carcinogenesis to establish the correlations. More recently, we have investigated similar relationships between target and nontarget cell populations within the liver. Chronic exposure to methylating hepatocarcinogens predominantly induces hemangiosarcomas, whereas exposure to ethylating agents causes hepatocellular carcinomas. This cell specificity in carcinogenesis correlates well with the presence of promutagenic DNA adducts. In the case of methylating agents, the nonparenchymal cells accumulate O⁶-methylguanine whereas the hepatocytes do not. Exposure to ethylating agents leads to accumulation of O⁴-ethyldeoxythymidine, but not O⁶-ethyldeoxyguanosine in hepatocytes. These differences reflect the ability of the two cell populations to repair O⁶-alkylguanine and the extent of purine and pyrimidine alkylation with methylating and ethylating agents. Hepatocytes of rats exposed to diethylnitrosamine for 28 days have four to five times more promutagenic DNA adducts (O⁶-alkyldeoxyguanosine and O⁴-alkyldeoxythymidine) than hepatocytes of rats exposed to nearly equimolar doses of dimethylhydrazine. Both O⁶-methylguanine and O⁴-methyldeoxythymidine are rapidly repaired by rat hepatocytes, while only O⁶-ethyldeoxyguanosine is rapidly repaired. Studies comparing the relationship between the induction of γ -glutamyl transpeptidase-positive foci, hepatocellular carcinoma and promutagenic lesions such as O⁴-ethyldeoxythymidine will be useful in understanding associations between the molecular dosimetry of DNA adducts, initiation, and progression of hepatocarcinogenesis.

During the past decade, relationships between chemical carcinogenesis and DNA adducts have been investigated for many classes of carcinogens. Most attention has focused on simple alkylating agents. Since many alkylating agents are tissue- or even cell-specific for cancer induction, comparisons between DNA adduct formation and repair in target and nontarget sites can be made. Data suggest that no single adduct is responsible for the initiation of chemical carcinogenesis. Rather, we propose that a balance between the formation and repair of all promutagenic DNA adducts and the extent of cell replication for each population of cells exposed determines the probability of neoplasia.

Correlations between DNA Adducts and Carcinogenesis in the Target Organ

While the N-7 position of guanine is known to be the most frequent site of alkylation, no correlation has been

found between 7-alkylguanine and tumor induction (1). In 1974, Goth and Rajewsky (2) and shortly thereafter Kleihues and Margison (3) demonstrated that O⁶-alkylguanine persists in rat brain, but not in liver or kidney, following exposure to ethyl or methylnitrosourea (MNU). Since these agents are potent neurocarcinogens, these data were heralded as strong evidence that O⁶-alkylguanine was the DNA adduct responsible for brain tumor induction. Further support for this was provided by studies showing accumulation and persistence of O⁶-methylguanine (O⁶-MG) in brains of rats exposed to MNU (15). Even though methyl methanesulfonate (MMS) was much less potent than MNU in inducing brain tumors, tumor induction was proportionate to the amount of O⁶-MG in brain (6). More recent investigations have shown, however, that persistence of O⁶-MG does not always result in the induction of brain tumors. Gerbil, hamster, and mouse brains develop concentrations of O⁶-MG similar to rats, yet have little or no susceptibility to brain tumor induction by MNU (7). Thus, mechanisms responsible for species differences in susceptibility to brain tumor induction by alkylating agents remain unknown.

Cell replication is an important factor in modulating the target site and incidence of tumor formation. For example, even though O⁶-MG is present in much greater

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concentrations in livers than in colons of rats exposed to 20 mg/kg dimethylhydrazine (SDMH), tumors occur in the colon, not in liver following weekly exposures. A major difference between these tissues is the extent of cell replication, being much greater in the colon. Thus, the amount of O⁶-MG per replicating cell was found to be greater in the target organ, the colon (8,9). Similar findings have been demonstrated in the replicating livers of newborn versus the minimally replicating livers of adult rats exposed to a single dose of dimethylnitrosamine (DMN), where tumors only occurred following exposure of the newborn (10). Recent studies by Dyroff et al. (12) demonstrated dramatic differences in the induction of hepatocellular carcinomas when 4-week-old versus 8-week-old F-344 rats were exposed to 40 ppm diethylnitrosamine (DEN) in their drinking water. The younger animals developed a 100% incidence of hepatocellular carcinomas after 6 weeks of exposure to DEN followed by 14 weeks additional exposure to phenobarbital, whereas none of the 8-week-old rats developed carcinomas when exposed to a similar regimen. Nearly a 10-fold greater number of γ -glutamyl transpeptidase-positive (GGT+) foci developed in livers of 4-week-old versus 8- or 12-week-old rats exposed to 40 ppm DEN for 4 weeks. This marked age effect may be due to the massive difference in percent body weight gain during DEN exposure, a crude index of cell proliferation. The 4-week-old rats underwent a 400% increase in body weight, compared to a 20% increase in the 8-week-old animals. As will be discussed in greater detail later, livers from both 4- and 8-week-old rats develop similar concentrations of O⁴-ethyldeoxythymidine ($\approx 1/10^5$ deoxythymidine). Thus, cell replication is probably a major factor influencing the induction of GGT+ foci and hepatocellular carcinoma in animals with similar concentrations of promutagenic DNA adducts.

Correlations between DNA Adducts and Carcinogenesis in the Target Cell

In addition to being organ specific in the induction of neoplasms, several alkylating agents are also selective for particular cell populations within that tissue. We have used this phenomenon to more critically evaluate the correlation between DNA adducts and carcinogenesis in the liver, where correlations between hepatocarcinogenesis and O⁶-alkylguanine were poor (10,11). Since whole liver is predominantly composed of hepatocytes, these correlations might be expected if tumors arose from cell types other than hepatocytes. The chemicals involved in our investigations included two procarcinogens (SDMH and DMN) that primarily induce angiosarcomas of the liver and one (DEN) that is selective for hepatocellular carcinoma induction. Our studies utilized dosing regimens similar to those employed in chronic bioassays, since it is the molecular dosimetry of chronic exposure that must be correlated with tumors induced by such regimens. In order to accomplish this,

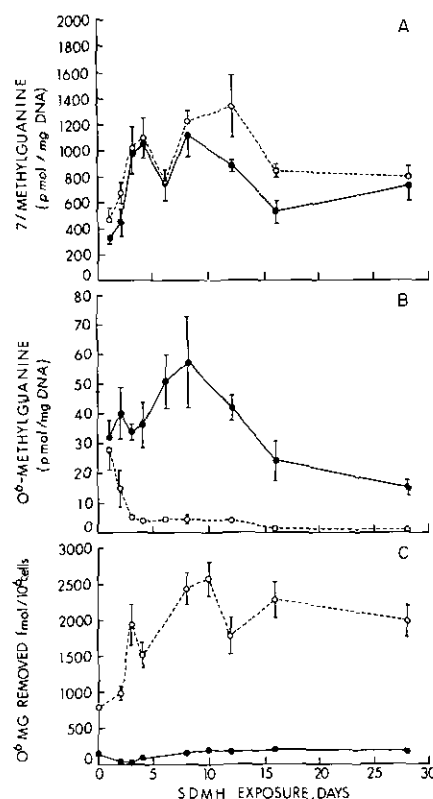


FIGURE 1. Normalized concentrations of (A) 7-MG and (B) O⁶-MG (13); and (C) O⁶-MGMT activity (19) in rat hepatocytes (---) and NPC (—) during exposure to SDMH (30 ppm) in the drinking water for up to 28 days. Data are expressed as means \pm SEM for three to six rats. O⁶-MGMT activity is expressed as fmole of O⁶-MG removed per 30 min per 10^6 cells.

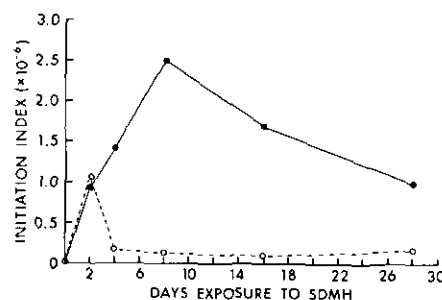


FIGURE 2. The initiation index for SDMH exposure of rats represents the probability of mutation due to O⁶-MG. Data points represent the product of O⁶-MG concentrations and *de novo* DNA synthesis per mg DNA \times the amount of DNA per cell population for (—) NPC and (---) hepatocytes (21).

DNA adducts were quantitated by using sensitive methods not requiring the administration of radioactive carcinogen. Studies on 7-alkylguanine and O⁶-methylguanine utilized HPLC separation coupled to fluorescence detection (13–16), while those on O⁶-ethyldeoxyguanosine and O⁴-alkylthymidine used radioimmunoassays (17,18). Following collagenase perfusion, livers were separated

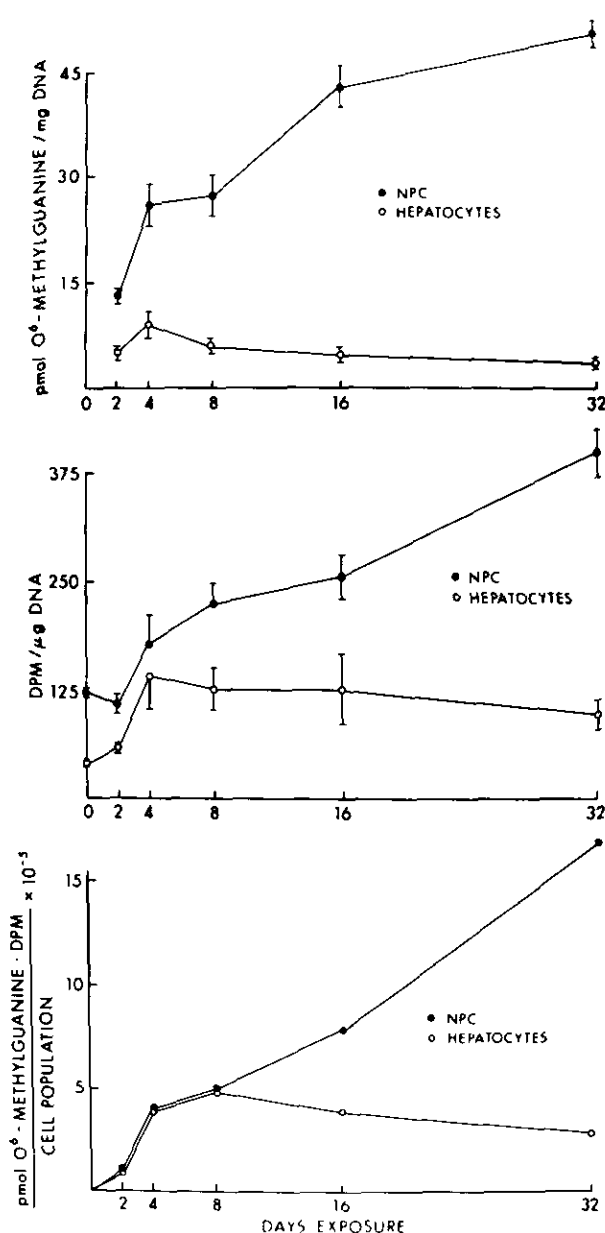


FIGURE 3. The product of (A) O⁶-MG concentration, (B) *de novo* DNA synthesis, and amount of DNA at risk per cell population have been used to calculate the initiation indices (C) for hepatocytes and NPCs of C3H mice during continuous DMN exposure (14).

into nonparenchymal cells (NPC), consisting of endothelial and Kupffer cells, and hepatocytes by either centrifugal elutriation (13,16) or low-speed centrifugation (14,17,18).

Exposure of F-344 rats to drinking water containing 30 ppm SDMH resulted in similar concentrations of 7-methylguanine (7-MG) in both NPC and hepatocytes (Fig. 1) (13). Steady-state concentrations of ~ 1000 pmole/mg DNA were attained as early as the third day of exposure. Both cell populations had similar O⁶-MG concentrations after one day of exposure; however, from that time on the O⁶-MG concentration in hepatocytes de-

creased. By the third day of exposure, hepatocyte O⁶-MG concentrations were ~ 4 pmole/mg DNA and by 16 days of administration concentrations had decreased to ~ 1 pmole/mg DNA. After 16 to 28 days SDMH exposure the O⁶/N-7 ratio was ~ 0.001. In contrast, the NPC accumulated O⁶-MG for the first 8 days, increasing from ~ 30 pmole/mg DNA on day 1 to ~ 57 pmole/mg DNA on day 8. O⁶-MG then began to decline, reaching a steady state of ~ 20 pmole/mg DNA between 16 and 28 days exposure. The O⁶/N-7 ratio of NPC was ~ 0.05 at this time.

The marked difference in O⁶-MG concentrations between NPC and hepatocytes was due to differences between the two cell populations in both the constitutive amount and the inducibility of the DNA repair enzyme, O⁶-methylguanine DNA methyltransferase (O⁶-MGMT). Control hepatocytes were shown to have ~ 5 times more O⁶-MGMT per cell than NPC (19). Furthermore, the O⁶-MGMT activity in hepatocytes was induced during exposure to SDMH. This induction correlated with the rapid decrease in O⁶-MG concentrations in hepatocytes. In contrast, the O⁶-MGMT activity in NPC was depleted during the initial 3-4 days of SDMH exposure, after which it returned to constitutive levels. Accordingly, the rapid accumulation of O⁶-MG in NPC corresponded with the depletion of O⁶-MGMT, while the subsequent decline in O⁶-MG corresponded with the return of O⁶-MG to constitutive levels.

Parallel investigations on *de novo* DNA synthesis demonstrated that the NPC underwent a marked mitogenic stimulus during SDMH exposure (20). Hepatocytes also showed increased cell replication; however, the extent was much less. If one examines the product of cell replication × the concentration of O⁶-MG during SDMH exposure and normalizes this with the amount of DNA per cell population, the area under each curve represents the theoretical probability of initiation per liver due to O⁶-MG (Fig. 2)(21). Using this calculation, the probability of inducing angiosarcomas is 17-fold greater than that of hepatocellular carcinomas. Furthermore, the predicted probability of hepatocellular carcinoma induction is highest in the first 4 days of SDMH exposure. A bioassay using the same dosing regimen demonstrated that angiosarcomas were induced in 93% of the rats, while hepatocellular carcinomas were produced in 40% of the animals (13). Thus, this model for initiation underestimated the latter, suggesting that DNA adducts other than O⁶-MG might be involved. As will be discussed in greater detail in subsequent portions of this review, it is likely that O⁴-methyldeoxythymidine also contributes to the initiation of hepatocytes (18).

Studies also have been conducted on C3H mice exposed to DMN (14). The NPC progressively accumulated O⁶-MG over 32 days of exposure to 10 ppm DMN, reaching ~ 50 pmole/mg DNA (Fig. 3). In contrast, the hepatocytes maintained constant amounts of O⁶-MG (~ 5 pmole/mg DNA) throughout the exposure regimen. Both cell populations exhibited a 2- to 3-fold increase in *de novo* DNA synthesis during the 32-day period of exposure to DMN. When these data were used to pre-

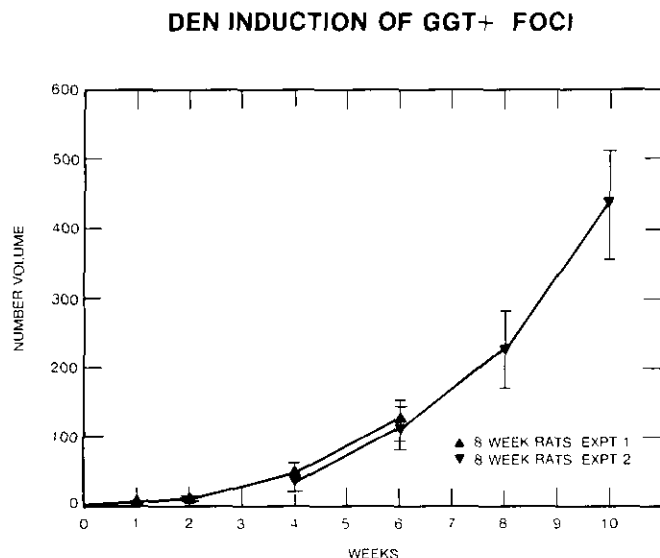


FIGURE 4. Hepatic GGT+ foci induction versus weeks of exposure to 40 ppm DEN in 8-week-old rats (12).

dict the likelihood of initiation due to O^6 -MG, the target cells (NPC) were predicted to be 2.5 times more likely to be initiated during the 32-day exposure than the hepatocytes, a finding in general agreement with several bioassays.

Similar methods were used to investigate the mechanisms of hepatocellular carcinoma induction by DEN. Initial studies using fluorescence detection for O^6 -ethylguanine (O^6 -EG) were hampered by concentrations of O^6 -EG too low to be detected by this method (22). Since O^6 -EG is known to be repaired in a manner similar to O^6 -MG, it was postulated that most initiation due to O^6 -EG would occur during the first 1 to 2 weeks of exposure. Therefore, if hepatocellular carcinoma induction by DEN was primarily due to O^6 -EG, initiation also should occur primarily during the first 2 weeks of exposure. This was tested by exposing rats to 40 ppm DEN in their drinking water for up to 10 weeks, followed by placing the animals on the Solt-Farber selection regimen for GGT+ foci (12). The data in Figure 4 clearly demonstrate that initiation was not confined to the first 2 weeks. Rather, the number of GGT+ foci remained similar to controls during the first 2 weeks and then progressively increased with increasing length of exposure. These data strongly suggested that DNA adducts other than O^6 -EG were responsible for hepatocellular carcinoma induction.

In collaboration with Manfred Rajewsky, we examined the molecular dosimetry of O^6 -ethyldeoxyguanosine (O^6 -EtdGuo) and O^4 -ethylthymidine (O^4 -EtdThd) during continuous exposure to DEN for up to 11 weeks (Fig. 5) (17). Using monoclonal antibodies in sensitive radioimmunoassays, we showed that O^4 -EtdThd accumulated in hepatocyte DNA to concentrations more than 50 times greater than O^6 -EtdGuo, in spite of its chemical formation in the DNA at only 1/3 to 1/4 the amount of O^6 -EtdGuo. Thus, the minor DNA adduct, O^4 -EtdThd,

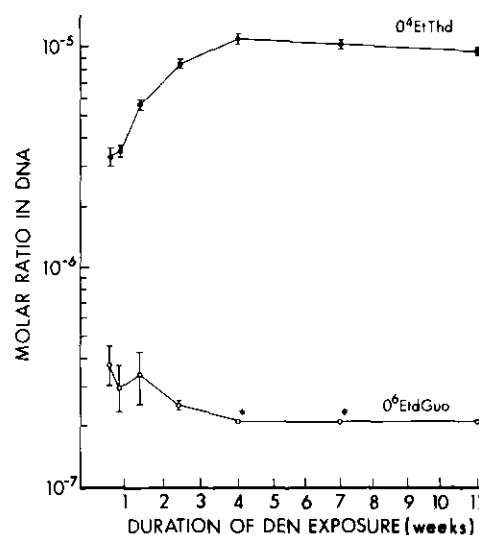


FIGURE 5. Concentrations of (●) O^4 -EtdThd and (○) O^6 -EtdGuo in DNA of hepatocytes of rats exposed to 40 ppm DEN in the drinking water for 2 days to 11 weeks. Data are expressed as means \pm SEM except points marked by an asterisk (*), where all samples were below the limit of detection (<200 fmole) (17).

was clearly a major promutagenic adduct in hepatocytes of rats continually exposed to DEN. After 2 days of DEN exposure, the NPC contained similar amounts of O^6 -EtdGuo and O^4 -EtdThd, being 1/3 that of the corresponding hepatocyte O^4 -EtdThd concentrations. At 49 and 77 days of exposure, the O^4 -EtdThd concentration in NPC remained about 1/2 that of the hepatocytes; however, the NPC O^6 -EtdGuo concentrations were 1/4 and 1/20 that of the O^4 -EtdThd, respectively.

Subsequent studies utilized polyclonal antibodies to O^4 -EtdThd to demonstrate that similar steady-state concentrations (~ 1 per 10^5 dThd) were achieved when 4-week-old F-344 rats were exposed to 40 ppm DEN (Fig. 6) (12). These animals were much more sensitive to the induction of GGT+ foci and hepatocellular carcinoma. Since the molecular dosimetry of 4- and 8-week-old rats was similar, this increased susceptibility most likely reflects increased cell proliferation associated with the rapid growth of these younger animals (12).

As shown in Figure 6, GGT+ foci appeared earlier and in greater number in 4-week-old rats, than in 8-week-old rats (Fig. 4)(12). Of greater interest was the demonstration that the number of foci per cubic centimeter plateaued. Since foci are believed to represent initiation of hepatocytes, which is a cumulative event, this was unexpected. The most likely interpretation of these data is that the plateau represents a steady state, during which initiated cells are being formed at a rate similar to that of their loss. Loss could be the result of a change in phenotype or cell death at the single cell stage of focus development.

Polyclonal antibodies to O^4 -methylthymidine (O^4 -MedThd) were also developed, permitting the first measurements of this minor alkylation product in animals exposed to methylating agents (18). Following a single

20 mg/kg exposure to SDMH, O^4 -MedThd was present in rat liver at 1/100 the amount of O^6 -MG. O^4 -MedThd was removed from rat liver relatively rapidly, having a half-time of ~ 20 hr (Fig. 7). In contrast, O^4 -EtdThd had a half-time of ~ 11 days, whether it was formed by a single exposure to 15 mg/kg DEN or by continuous exposure to 40 ppm DEN in the drinking water for 4 weeks. No evidence for an inducible repair system for O^4 -EtdThd was demonstrable. Thus, differences in repair rates of O^4 -MedThd and O^4 -EtdThd provides a mechanism to explain the greater ability of ethylating versus methylating agents to induce hepatocellular carcinoma in the rat. This is shown in Table 1, where alkylation data from hepatocyte DNA of rats exposed to equimolar doses of SDMH and DEN for up to 28 days were compared (18). The data presented are accumulations of O^6 -alkylguanine, O^4 -alkylthymidine, and the sum of these two promutagenic DNA adducts at various times of exposure and therefore represent the net effects of formation and repair. Under these conditions, O^6 -MG attained steady-state concentrations ~ 10 times higher than O^6 -EG, while O^4 -MedThd plateaued at concentrations 10 times lower than O^4 -EtdThd. Since nearly equal amounts of O^4 -alkylthymidine were formed chemically, it is apparent that the difference in the rate of repair is the predominant factor leading to higher levels of O^4 -EtdThd versus O^4 -MedThd. In contrast, differences in formation are the principal factors leading to higher levels of O^6 -MG vs. O^6 -EG. It is also of interest to note that even though O^4 -MedThd is formed at only 1/100 the amount of O^6 -MG, it accumulates to nearly equal concentrations in hepatocyte DNA of rats continuously exposed to SDMH. These data clearly show that the molecular dosimetry of DNA adducts resulting from single dose studies are a poor surrogate for data from chronic exposure regimens. One must match bioassay data with molecular dosimetry data derived from comparable dosing regimens.

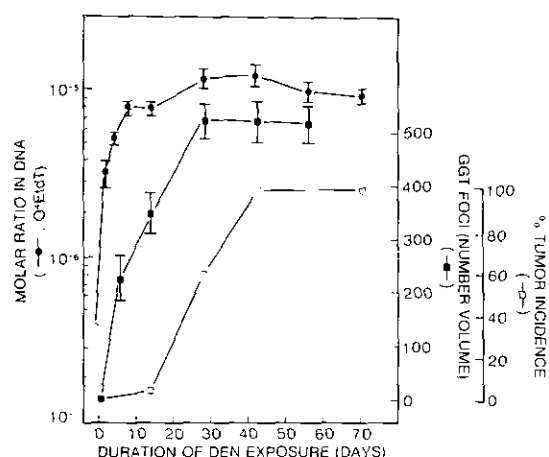


FIGURE 6. Correlation of (●) O^4 -EtdThd persistence with (■) hepatic GGT+ foci and (□) tumor induction in 8-week-old rats (12).

Potential of Molecular Dosimetry in Risk Assessment

The field of risk assessment is rapidly changing from presenting qualitative risk assessments to making quantitative estimates of risk. Numerous mathematical models have been developed, ranging from linear extrapolation based on a one-hit one-cancer theory, to present day multistage and multihit models thought to better approximate the steps involved in carcinogenesis. A critical problem is that the accuracy of these models can not be validated with bioassay data. Data from the ED₀₁ study, which involved exposure of 24,192 mice to 2-acetylaminofluorene, have been used but are woefully inadequate for low-dose extrapolation, since the dose range employed covered less than one order of magnitude (23). This study points out the futility of conducting animal bioassays to validate true low dose extrapolations (four to six orders of magnitude), since millions of animals would be required to detect a response at such extremely low doses. An alternative method using molecular dosimetry as the measure of exposure followed by an examination of tumor incidence relative to DNA adducts has been suggested. Present-day models for quantitative risk assessment use measures of external exposure such as ppm in air, water, or diet. Such measures of exposure cannot account for nonlinearities in the dose response (24,25). The schematic shown in Figure 8 shows some of the possible fates of external exposure. Absorption of a chemical can range from 0 to 100%, yielding the internal exposure or dose. This internal dose can be metabolized to an activated or a detoxified form, again ranging from 0 to 100%, yielding the internal activated dose. This dose is distributed to critical and noncritical sites. By measuring the dose at target sites, one should have the most

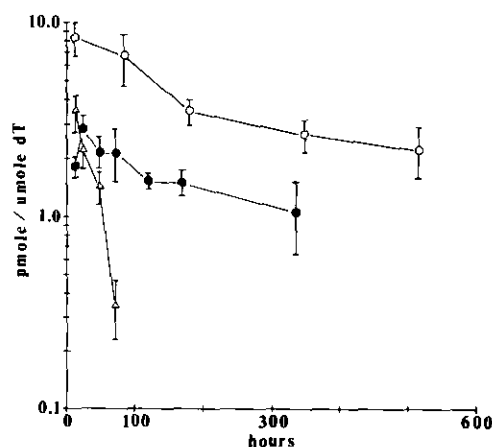


FIGURE 7. Persistence of O^4 -EtdThd and O^4 -MedThd following pulse (●) DEN or (Δ) SDMH or (○) continuous DEN administration. The curve for O^4 -EtdThd following continuous DEN has been shifted 12 hr to correspond with 12 hr time points in pulse dosing regimens. All control liver values were below the limits of detection. Data are expressed as means \pm SEM for three to six rats (18).

Table 1. Concentrations of O⁶-alkylguanine and O⁴-alkyldeoxythymidine in hepatocyte DNA of rats exposed to 40 ppm DEN or 30 ppm SDMH in drinking H₂O.^{a,b}

Exposure period, days	Carcinogen	Concentration of O ⁶ -alkyl G, pmole/μmole G	Concentration of O ⁴ -alkyl dT, pmole/μmole dT	Total concentration of O ⁴ -alkyl dT + O ⁶ -alkyl G pmole/μmole DNA ^c
8	DEN	0.3	5.0	2.0
	SDMH	6.3	0.8	1.6
16	DEN	0.25	8.5	2.4
	SDMH	1.6	0.8	0.6
28	DEN	<0.2	10.0	2.8
	SDMH	1.6	1.0	0.6

^aData of Richardson et al. (18).

^bThe exposures result in ~29 μmole DEN and 34 μmole SDMH per kg per day.

^cSince dT/dG ratios ranged from 1.28 to 1.35, 1 μmole DNA contains 0.22 μmole dG and 0.28 μmole dT. The sums expressed are therefore adjusted accordingly.

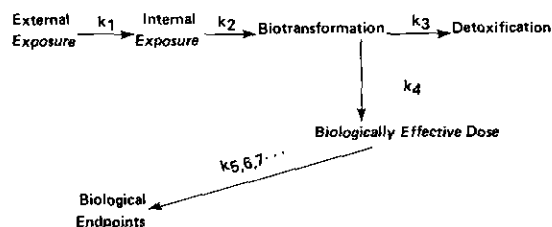


FIGURE 8. Relationship between external exposure and biologically effective dose.

accurate data for determining the true dose response relationship. DNA adducts represent a critical target site dose that can be utilized for such studies. As the dose-response relationship is established, molecular dosimetry may extend the observable range of biological data. Even when it becomes impractical to obtain tumor data due to the large numbers of animals needed, the relationships between external exposure and target site dosimetry can be determined. Furthermore, this relationship can be compared across species, including exposed humans. The inclusion of such data could place quantitative risk assessment on a much firmer scientific base than is presently available. Scientists conducting studies on DNA adducts are therefore encouraged to provide their counterparts in risk assessment with data suitable for developing models based on molecular dosimetry.

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